

Milk-Clotting Enzymes From Microorganisms

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ABSTRACT

SRINIVASAN, R. A. (National Dairy Research Institute, Karnal, India), M. K. K. IYENGAR, I. J. BABBAR, S. C. CHAKRAVORTY, A. T. DUDANI, AND K. K. IYA. Milk-clotting enzymes from microorganisms. *Appl. Microbiol.* **12**:475-478. 1964. —A total of 230 cultures of fungi and 43 cultures of bacteria, isolated from such sources as soil, butter, and milk, were screened for their milk-clotting activity. The fungi were cultivated on semisolid media, and the bacteria were grown in milk media in shake culture. Phytic acid, added as calcium phytate, was found to stimulate production of the enzyme in most of the bacterial isolates. Proteolytic activity was invariably found to be associated with the milk-clotting enzyme in bacterial isolates. There was considerable variation in the ratio of the two enzymes from strain to strain.

Investigations on the milk-clotting enzymes have included plant materials such as *Withania coagulans* (Dastur, Sastry, and Venkatapiah, 1948), *Ficus carica* (Whitaker, 1959; Krishnamurti and Subrahmanyam, 1948; Zuckerman-Stark and Leibowitz, 1961, 1963a, b), *Carica papaya* (Dastur, 1949), *Cynara* species (Russo, 1961), and pumpkin (Rebecca and Leibowitz, 1963). Various fungi, particularly Entomophthorales (Oringer, 1960; Whitehill et al., 1960), are potent sources suggested for cheese making. The bacteria investigated for this purpose are *Bacillus brevis*, *B. cereus*, *B. mesentericus*, *B. subtilis*, and *Streptococcus liquefaciens* (Shimwell and Evans, 1944; Srinivasan et al., 1962a, b; Emanuiloff, 1956). Microorganisms are known to be highly versatile in producing a wide range of enzymes, with varied patterns of activity. The present report deals with the screening of various fungi and bacteria for the production of milk-clotting enzymes and (in a few cases) proteolytic enzymes. The effect of calcium phytate on the production of milk-clotting enzymes was also studied.

MATERIALS AND METHODS

Isolation of fungi. Samples of soil, butter, khoa, cheese, and moldy vegetables were plated on two media, Potato Dextrose Agar (Difco) and Czapek Dox Agar (BBL). These media were supplemented with 1% sterile skim milk at the time of plating. The plates were inoculated with the samples, incubated for 3 days at 30 C, and distinct colonies were isolated. The isolated colonies were streaked on slants at 30 C; after abundant growth of the cultures,

the tubes were stored at room temperature. The cultures were subcultured every 5 to 6 weeks.

Isolation of bacteria. Samples of soil, raw and boiled milk, etc., were plated on Tryptone (Difco) media containing skim milk. Suitable dilutions of test material were plated and incubated at 37 C for 48 hr. Bacterial cultures, particularly those showing a clear zone, were transferred to slants of the same medium at 37 C, and were then stored in a refrigerator (5 C). The isolates were subcultured every 6 weeks.

Screening for milk-clotting enzymes. The mold isolates were transferred to the medium of Gastrock et al. (1938), in which beer was replaced by 0.5 g of yeast extract per 100 ml of medium, and were incubated for 3 days at 30 C. Saline suspension of the growth was used for inoculation into sterilized semisolid medium (10 g of wheat bran, 0.1 ml of fresh milk, and 10 ml of distilled water). The molds were cultivated at 30 C for 5 days. The growth was then extracted with 50 ml of saline, and the milk-clotting activity was determined.

In the bacterial screen, 100 ml of fresh milk (pH about 6.5) were added to 500-ml Erlenmeyer flasks, which were then sterilized by autoclaving at 15 psi for 20 min. The flasks were cooled to about 30 C, and were inoculated with a saline suspension of bacterial isolates grown for 24 hr. The organisms were cultivated for 72 hr under submerged and surface conditions. In submerged culture, the inoculated flasks were kept at 30 C on a rotary shaker (about 200 rev/min). In surface culture, the flasks at 30 C were not disturbed during the period of growth. Most of the organisms were pellicle-formers in surface cultures, and hence it was assumed that the majority of the cells were retained on the surface of the medium.

Assay for milk-clotting activity. Pasteurized milk heated at 71 C for 1 min and immediately cooled and fortified with 0.2% calcium chloride was used as substrate. For assay of enzyme activity, 1 ml of the broth of a suitable dilution (at 40 C) was added to 5 ml of whole milk (also at 40 C). The broth was diluted with distilled water. Under these conditions, the time of clot was found to be inversely proportional to enzyme activity, except for higher than fivefold dilutions of the broth. Milk-clotting activity was calculated as follows: in liquid cultures, activity per milliliter of broth is represented by $60/t \times DF$, where t is the clotting time in seconds and DF is the dilution factor. Mold bran activity per gram is expressed as $60/t \times 1/g \times$

DF, where g is the amount in grams of mold bran taken for analysis.

Assay for proteolytic activity. The bacterial broths used as the source of proteolytic enzyme were filtered through a Seitz filter. The clear filtrates were diluted 1:10 with phosphate buffer (pH 6.0; final concentration, 0.1 M). The substrate employed for the assay of proteolytic activity was soluble casein (Hammersten). The casein solution was prepared by the method of Kunitz (1947): 1 ml of diluted enzyme solution was added to 2 ml of casein solution (pH 6.0) and 1 ml of 1.0 M phosphate buffer (pH 6.0); the resultant solution was mixed well and kept at 40 C for 20 min. Then, 5 ml of 5% trichloroacetic acid were added, and the mixture was allowed to stand for 30 min at room temperature. The resultant precipitate was removed by filtering the solution through Whatman no. 4 filter paper. The concentration of split products in the filtrate was determined essentially by the method of Layne (1957). To 2 ml of filtrate, 4 ml of alkaline copper sulfate solution and 0.4 ml of distilled water were added. After 10 min, 0.1 ml of diluted Folin's reagent was added, and the mixture was held for 30 min or longer for development of color. The optical density was read at 750 mμ in a Beckman spectrophotometer (model DU). Optical density expresses enzyme activity.

RESULTS AND DISCUSSION

More than 90% of the isolates of molds were from soil or butter; the main source of bacterial isolates was heat-treated milk (Table 1). Of the isolates in Table 1, 210 strains of molds and 40 strains of bacteria were screened for their milk-clotting activity. The molds were classified into three groups, according to their activity.

The mold isolates in group I (Table 2) exhibited activity ranging from 10 to 20 units, with an average of 13 units. This activity was almost twice that shown by members of

group II (range of 5 to 9 units, and average of 7 units) and about four times the activity of group III (range of 2.0 to 4.4 units, with an average of 3.5 units). Group I invariably yielded curd with loose texture, whereas groups II and III gave curds with either loose or firm texture. It is known that milk-clotting activity is invariably accompanied by proteolytic activity. Bargoin (1963) believed the two enzymes to be independent, based on their behavior on dephosphorylated casein. At the moment of clotting, there is a release of some peptide bond. This release seems to originate from K-casein (Wake, 1959). The extent of the release is likely to be different in enzyme preparations obtained from various sources; for example, rennet has one bond per molecular weight of 1,200,000 (Nitschmann and Varin, 1951), but trypsin has many more. Because of the considerable proteolysis concurrent with milk-clotting, the observance of clot may be entirely missed or the clot obtained may be loose. The cause of loose clot invariably shown by group I molds may thus be due to high proteolytic activity of the molds.

The bacterial isolates were screened on milk medium in surface as well as submerged conditions of growth. The bacterial strains which yielded appreciable milk-clotting activity are reported in Table 3. These organisms produced a low concentration of milk-clotting enzyme

TABLE 1. Isolates of milk-clotting microorganisms

Source	No. of samples	Mold isolates	Bacterial isolates
Soil	116	196	—
Butter.....	17	19	—
Cheese	1	4	1
Condensed milk	3	5	—
Channa.....	2	1	—
Milk (heat-treated at 100 C for 15 min).....	33	—	37
Raw milk.....	3	—	3
Miscellaneous.....	7	6	2
Total.....	182	231	43

TABLE 2. Summary of the screening of molds

Group	Activity (units per g of mold bran)	No. of mold isolates	Percentage of total
Group I (high activity)	10-20	12	34.3
Group II (medium activity)	5-8	12	34.3
Group III (low activity)	2-4	11	31.4

TABLE 3. Milk-clotting activity of some bacterial isolates

Bacillus isolate no.	Activity (units) per ml of broth (72 hr)	
	Surface culture	Submerged culture
1	0.3	0.500
2	0.2	0.500
3	0.3	0.100
4	0.5	0.250
5	0.2	0.062
6	0.2	0.200
7	<0.006	0.170
8	<0.006	0.170
9	0.01	0.250
10	1.2	4.000

TABLE 5. Milk-clotting and proteolytic activity in bacterial isolates

Bacillus isolate	Milk-clotting activity	Proteolytic activity	Milk-clotting activity-proteolytic activity ratio
K ₁	0.60	0.0055	109.0
K ₂	0.22	0.0025	88.0
K ₃	2.00	0.0900	22.0
K ₄	1.20	0.025	48.0
K ₁₁	1.66	0.015	110.6
K ₁₂	1.50	0.020	75.0
K ₁₆	1.36	0.065	20.9
K ₁₉	0.65	0.050	13.0
K ₂₆	10.00	0.068	148.0
K ₂₉	3.33	0.070	47.5
K ₃₀	1.32	0.075	17.6
K ₃₁	0.46	0.040	11.5
K ₃₃	0.68	0.028	24.7
K ₃₆	1.00	0.043	23.5
K ₃₉	0.46	0.045	10.2

under both surface and submerged conditions. The maximal enzyme activity was 0.5 units, with the exception of isolate 10 which gave an enzyme activity of 4.0 units on submerged culture. In a few cases, there was an appreciable increase in production under submerged culture compared with surface culture. This was particularly true for cultures 2, 7, 8, and 10. A decrease in enzyme production in submerged culture was noticeable, however, with isolates 3, 4, and 5. Although milk medium was a poor medium for the production of milk-clotting enzyme, it served as a good means of preliminary screening of bacterial strains.

Effect of calcium phytate on milk-clotting enzyme. Phytic acid and calcium phytate have been reported to stimulate the production of α -amylase of *Bacillus subtilis* (Dunn et al., 1959) and protease of some bacteria (Tsuychihiara 1954). We therefore studied the effect of calcium phytate on the production of milk-clotting enzyme by some bacterial isolates. Sterilized fresh milk was employed as the basal medium, to which calcium phytate was incorporated at a concentration of 0.2 or 0.4%. Control medium consisted of milk and calcium chloride, calcium being in molar concentration equal to that of calcium phytate. The influence of calcium phytate on the production of the enzyme by eight bacterial isolates is given in Table 4.

On the basis of the effect of calcium phytate on the production of milk-clotting enzymes, the various spore-forming bacterial isolates could be classified into three categories. In the first category was isolate H, which neither produced milk-clotting enzyme in the basal medium nor when basal medium was supplemented with calcium phytate. The second category included six isolates (A-F), which did not produce the enzyme in the basal medium but elaborated appreciable amounts when calcium

phytate was incorporated into it. In the third group (isolate I), an appreciable amount of the enzyme was produced in the basal medium, but calcium phytate adversely affected the production of enzyme.

Milk-clotting and proteolytic activities of bacteria. Milk-clotting enzymes are invariably accompanied by proteolytic activity. In a program for the screening of spore-forming bacteria for milk-clotting activity, it was of interest to determine their proteolytic activity (exocellular). The results obtained with a few promising strains are given in Table 5.

To compare the extent of production of the two types of enzyme activities in the broths of various sporeforming bacterial isolates cultivated in submerged culture, the milk-clotting activities are taken as the basis, and the elaboration of corresponding proteolytic activities are discussed. The main question is whether equal amounts of milk-clotting activity produced by various isolates are accompanied by equal amounts of proteolytic activity. This would mean that such organisms would exhibit an equal ratio of milk-clotting activity to proteolytic activity. The ratio of milk-clotting to proteolytic activity is referred to as the *index* for the organism. The bacteria examined were classified into two groups.

Group I includes those isolates which showed good agreement in the extent of production of the two enzymes. Isolates K₃₁, K₃₉, K₁₆, and K₃₀ fall into this group. These organisms produced equal milk-clotting and proteolytic activities, and hence had an index of 1. In some organisms, such as K₃ and K₃₆, the multiples of milk-clotting activity ($\times 2$) were equal to proteolytic activities ($\times 2$). K₃ had milk-clotting activity about twice that of K₃₆, and also had proteolytic activity about twice that of K₃₆.

In group II, there was pronounced disagreement in the

TABLE 4. Effect of calcium phytate on production of milk-clotting enzyme*

Bacillus isolate	Age of culture	Milk only		Milk plus 0.2% calcium phytate		Change over control	Milk plus 0.4% calcium phytate		Change over control
		pH	Activity	pH	Activity		pH	Activity	
	hr		units/ml		units/ml			units/ml	
A	48	7.1	NC	7.1	0.110	+0.110	7.1	0.143	+0.143
	72	7.4	NC	8.0	0.143	+0.143	8.0	0.120	+0.120
B	48	7.2	NC	7.1	1.000	+1.000	7.2	0.250	+0.250
	72	7.4	NC	8.2	0.300	+0.300	8.3	0.250	+0.250
C	48	5.8	NC	5.6	1.000	+1.000	7.0	0.520	+0.52
	72	5.7	NC	5.7	1.000	+1.000	8.2	0.330	+0.33
D	48	7.0	NC	7.0	0.250	+0.250	7.0	0.250	+2.250
	72	7.5	0.066	8.0	0.250	+0.250	7.6	0.200	+0.200
E	48	7.1	NC	7.1	0.071	+0.071	7.2	0.330	+0.330
	72	7.6	NC	8.0	0.110	+0.110	8.0	0.200	+0.200
F	48	7.1	NC	7.1	0.071	+0.071	7.2	0.330	+0.330
	72	7.8	NC	8.0	0.110	+0.110	8.0	0.200	+0.200
G	48	7.2	NC	7.1	NC	—	7.2	NC	—
	72	7.6	NC	8.0	NC	—	8.0	NC	—
H	48	5.7	0.280	5.7	1.660	+1.380	5.7	1.560	+1.280
	72	5.7	0.340	5.8	1.660	+1.320	5.7	0.530	+0.190
I	48	5.6	1.2	5.6	1.43	+0.23	5.6	0.83	-0.37
	72	5.8	1.43	5.6	1.20	-0.23	5.7	0.65	-0.78

* Calcium chloride did not influence enzyme production. NC = no clotting.

production of milk-clotting and proteolytic activities. In isolates K₁, K₁₉, and K₃₃, the milk-clotting activities were almost equal, but the differences in the proteolytic activities elaborated by them were very significant; their indices differed considerably, being 110.63, 13.0, and 24.7, respectively. For equal milk-clotting activity of about 0.6, the proteolytic activity of 0.0055 unit in K₁ was about one-tenth the proteolytic activity of K₁₉ and about one-fifth of K₃₃.

Isolate K₄ had 1.2 units of milk-clotting activity and 0.025 of proteolytic activity. This, by a multiple of 2, would have shown 2.4 units as milk-clotting activity and 0.05 units as proteolytic activity; K₃₃, which had 2.4 units as milk-clotting activity (equal to multiple of 2 of K₄), showed 0.075 unit of proteolytic activity, which was 1.5 times the theoretical for K₄ proteolytic activity.

Comparing K₂₆ and K₃₆, we found that the milk-clotting activity of K₃₆ when multiplied by 10 would equal the milk-clotting activity of K₂₆. The corresponding proteolytic activity in K₃₆ should theoretically also be a multiple of 10, but actually it corresponded to a multiple of 16.

A similar discrepancy existed in K₂ and K₃₉. For 0.22 unit of milk-clotting in K₂, proteolytic activity was 0.0025. In K₃₉, milk-clotting was 0.46, a multiple of about 2 of K₂ activity; proteolytic activity of K₃₉ was 0.045, which is about 10 times what it theoretically should have been (0.005) in the case of K₂.

From the above, it may be deduced that milk-clotting and proteolytic enzymes are in all probability different. This may confirm the finding of Bargoin (1963). Attempts to separate these activities have been reported in some plant materials, such as papaya latex (Jansen and Balls, 1941; Balls and Lineweaver, 1939), fig latex (Whitaker, 1959), and papain (Bahadur and Kumari, 1959); attempts with microbial preparations, such as *Streptococcus zymogenes* (Grutter and Zimmerman, 1955) and coccus P (Gorni and Lanzavecchia, 1954), have not been successful.

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